The Identification and Antimicrobial Susceptibility of Anaerobic Bacteria from Pneumonic Cattle Lungs

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ABSTRACT

One hundred and forty-four lungs obtained postmortem from cattle with pneumonia were cultured for anaerobic bacteria. Forty-five lungs yielded 73 anaerobic isolates belonging to 20 species. The number of isolations of anaerobes from acute fibrinous or suppurative bronchopneumonias (32.5%) was slightly lower than from similar chronic bronchopneumonias (36.5%). Anaerobes were not recovered from 15 lungs showing macroscopic changes not of bacterial origin, nor from 13 healthy lungs. The predominant genera isolated were *Bacteroides, Peptococcus, Fusobacterium* and *Clostridium*. The most common species were *P. indolicus* (15 isolates), *B. asaccharolyticus* (nine), *F. necrophorum* (six), *C. perfringens* (four) and *B. fragilis* (four). There was a significant correlation between the presence of *Corynebacterium pyogenes* ($p < 0.001$) or *Escherichia coli* ($p < 0.01$) and the presence of anaerobes in the lungs.

The isolated anaerobic bacteria were generally susceptible to ampicillin, penicillin G, cefoxitin, cephalothin, clindamycin, chloramphenicol, erythromycin, tetracycline and metronidazole. The *B. fragilis* and *C. perfringens* isolates showed multiple antibiotic resistance, and five *P. indolicus* isolates were resistant to tetracycline.

Key words: Cattle, pneumonia, anaerobic bacteria, antimicrobial susceptibility.

RÉSUMÉ

Cette étude consistait à rechercher des bactéries anaérobies dans les poumons de 144 bovins dont on venait d'effectuer la nécropsie. On en isola 73 souches qui appartenaient à 20 espèces, chez 45 de ces bovins; 32,5% des cas de bronchopneumonie fibrineuse ou purulente permirent d'isoler de ces bactéries, comparativement à 36,5% de ceux de bronchopneumonie chronique. Quinze échantillons pulmonaires dont les lésions macroscopiques différaient de celles d'une pneumonie bactérienne, ainsi que 13 autres échantillons apparemment sains, ne recelaient pas de bactéries anaérobies. On isola le plus souvent les genres suivants: *Bacteroides, Peptococcus, Fusobacterium* et *Clostridium*. Quant aux espèces, on identifia *P. indolicus* 15 fois, *B. asaccharolyticus* neuf fois, *F. necrophorum* six fois, *C. perfringens* et *B. fragilis* quatre fois chacune. On enregistra aussi une corrélation significative entre la présence de *Corynebacterium pyogenes* ($p < 0,001$) ou d'*Escherichia coli* ($p < 0,01$) et celle d'anaérobies.

Les souches de bactéries anaérobies ainsi isolées se révélèrent généralement sensibles aux antibiotiques suivants: ampicilline, pénicilline G, céfoxitine, céphalothine, clindamycine, chloramphénicol, érythromycine, tétracycline et métronidazole. Les souches de *B. fragilis* et *C. pyogenes* manifestèrent une résistance envers plusieurs antibiotiques, tandis que cinq souches de *P. indolicus* s'avérèrent résistantes à la tétracycline.

Mots clefs: bovins, pneumonie, bactéries anaérobies, sensibilité aux antibiotiques.

INTRODUCTION

Pneumonia is an important cause of economic loss in cattle (1). Several viruses and aerobic bacteria such as *Pasteurella haemolytica* and *P. multocida* are well established as aetiological agents of infectious pneumonia in cattle (2,3). The possible contribution of anaerobic bacteria to the pneumonic process in cattle has not been studied, although anaerobic bacterial lung infections are common and important in man (4). Such scanty veterinary reports as there are suggest the occasional involvement of anaerobic bacteria in bovine pneumonias (5,6).

The purpose of this study was to

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investigate the presence, identity and antimicrobial drug susceptibility of anaerobic bacteria from pneumonic cattle lungs.

MATERIALS AND METHODS

LUNG SAMPLES

A total of 172 bovine lungs was studied. One hundred and forty-four lungs were obtained postmortem from pneumonic cattle, autopsied by the Department of Pathology, University of Guelph, or by the Veterinary Services Branch, Ontario Ministry of Agriculture and Food, Guelph. The description of the pathological change in the lungs was based on both gross and microscopic appearance and is shown in Table I. The cattle had been dead for up to 48 hours. Any specimens which appeared grossly autolytic were not processed. Fifteen specimens were taken postmortem from animals whose lungs showed macroscopic changes (e.g. edema, congestion) not attributed to bacterial invasion of the lung tissue, e.g. disseminated intravascular coagulation or persistent *ductus arteriosus*. Thirteen specimens were taken from slaughtered animals showing no macroscopic changes in their lungs at postmortem. One section of lung of approximately $8 \times 5 \times 5$ cm was taken from an area showing gross pneumonic change, tightly wrapped in polyethylene bags and stored at -70°C until cultured.

CULTURE MEDIA

Columbia blood agar,[1] supplemented with vitamin K, 10 μg/mL and hemin, 5.0 μg/mL (CBA plates), was used for the isolation of anaerobic bacteria. The same medium was modified by the addition of kanamycin (100 μg/mL) and vancomycin (7.5 μg/mL) (KVBA plates) and used to selectively recover Gram-negative anaerobes. Details on the preparation and use of vitamin K, hemin, kanamycin and vancomycin are given elsewhere (7). The plates were prereduced by placing them in anaerobic jars immediately after preparation. Media used for aerobic cultures were trypticase soy agar[1] containing 5% bovine blood (BA plates) and MacConkey agar.[1] Peptone yeast glucose broth and chopped meat carbohydrates broth, prepared as described elsewhere (8), were used for analysis of the end fermentation products by gas-liquid chromatography (GLC). Supplemented brain-heart infusion broth, prepared as described elsewhere (9, 10), was used for broth-disc antimicrobial susceptibility tests.

CULTURE TECHNIQUES

Samples were thawed and a piece of tissue of approximately one cm^3 was taken aseptically and ground in a sterile Ten-Broeck grinder, with 2 to 5 mL of dilution fluid (6), previously boiled for ten minutes to expel dissolved oxygen. Two drops of the homogenate were plated on each medium (CBA, KVBA, BA and MacConkey agar plates). The BA and MacConkey plates were incubated aerobically at 37°C for three days. The CBA and KVBA plates were incubated at 37°C anaerobically. The anaerobic environment was established in standard anaerobic jars using either a commercial anaerobic culture system[2] or an evacuation replacement system, using a gas mixture consisting of 80% nitrogen, 10% carbon dioxide and 10% hydrogen, to fill jars which had been evacuated to 25 cm of mercury on four occasions. Jars were equipped with a catalyst of palladium-coated asbestos pellets which were reactivated after each use. Methylene blue indicator strips were always included in jars to confirm anaerobiosis.

In order to minimize exposure to air during processing, anaerobic jars containing prereduced plates were constantly flushed with carbon dioxide by means of a pipette connected to an oxygen-free carbon dioxide source. The carbon dioxide was passed over copper, heated to about 300°C in a thermostatically controlled oven, to remove traces of oxygen. The plates were removed for inoculation and immediately afterwards placed in another vented jar connected to the oxygen-free carbon dioxide.

Anaerobic jars were opened after 48 hours of incubation and the plates were examined under a stereoscopic microscope (15x). The different colony types observed were reisolated on CBA plates and the original plates were reincubated anaerobically for a further five days to ensure culture of slow-growing bacteria. All colony types isolated anaerobically were checked for aerotolerance; those growing aerobically were discarded.

IDENTIFICATION

Anaerobic bacteria were identified by a combination of identification of end products of fermentation by GLC, and by biochemical tests using a commercial miniaturized anaerobic identification system[3](9). For the GLC analysis, the procedures used and the interpretations of the results followed those of the Virginia Polytechnic Institute (8). Aerobic bacteria were identified by standard procedures (11, 12).

ANTIMICROBIAL SUSCEPTIBILITY TESTS

Two different methods were used to test the susceptibility of anaerobic isolates to 10 antimicrobial drugs.

The first method used commercially available frozen antimicrobial solutions contained in plastic trays,[4] which measured the minimal inhibitory concentrations (MIC) of the drug for the anaerobic isolates tested. The drugs tested are shown in Table IV. Panels

[1]Difco Laboratories, Detroit, Michigan.
[2]Gaspak envelopes; BBL, Becton Dickinson, Mississauga, Ontario.
[3]Minitek Anaerobe Numerical Identification System; BBL.
[4]Anaerobe MIC panels; Micromedia Systems, Potomac, Maryland.

were stored at -20°C until used; panels were always tested for potency using bacteria of known MIC (*Bacteroides thetaiotaomicron* ATCC 29741 and *Staphylococcus aureus* ATCC 29213) in parallel with the anaerobic isolates of unknown MIC.

A second method of determining susceptibility was used when it was found that the broth in the commercial panel method for determining MIC would support the growth of only half the isolates. This was the semi-quantitative broth-disc method of Wilkins and Thiel (10). The method involves the use of antibiotic susceptibility discs[1] as a source of drug, using a broth and conditions which support the growth of anaerobic bacteria. The antimicrobial drugs tested and their concentration is shown in Table V.

RESULTS

ISOLATION AND IDENTIFICATION OF ANAEROBIC BACTERIA

The pneumonic processes were classified on the basis of macroscopic and microscopic changes into six groups; Table I shows the number and percentages of each group yielding anaerobic bacteria. Lungs showing acute or chronic bronchopneumonia gave similar recovery of anaerobic bacteria, all of which were present in moderate or large numbers; 32.5% of acute bronchopneumonias and 36.5% of chronic bronchopneumonias yielded anaerobic bacteria. A total of 45 of 144 (31.2%) of the lungs showing macroscopic pathological change yielded anaerobic bacteria; 73 anaerobes were isolated from these 45 cases. The identity of these anaerobes is shown in Table II. These isolates belonged to 20 different species, grouped in seven genera. The most frequent genera isolated were *Bacteroides* (29 isolates, 39.7%) and *Peptococcus* (19 isolates, 26%). The predominant species isolated were *P. indolicus* (15 isolates) and *B. asaccharolyticus* (nine isolates). They were found respectively in 33.3% and 20.5% of lungs yielding anaerobes.

No anaerobic bacteria were isolated from 15 lungs with postmortem macroscopic changes not due to bacterial infection, nor were they recovered from 13 lungs of healthy cattle at slaughter.

From the 45 lungs yielding anaerobes, 95 facultative bacteria were isolated (Table III). They belonged to 13 different species grouped in 11 genera. The predominant species isolated were *Corynebacterium pyogenes*, *Escherichia coli*, *Pasteurella multocida* and *Pasteurella haemolytica*. In 43 of 45 cases when anaerobes were recovered, aerobic bacteria were also present. Analysis using Yates modification of the chi-square test showed a significant association between the presence of *C. pyogenes* ($p<0.001$) or *E. coli* ($p<0.01$) and anaerobic bacteria. There was no significant relationship between individual aerobic and anaerobic bacterial species, nor between the presence of individual anaerobic species. Of the 45 cases yielding anaerobic bacteria, 20 were animals aged one day to six months, 12 were animals six months to two years of age, and six were animals over two years. Age was not recorded for seven animals.

Of the 99 lungs not yielding anaerobic bacteria, 69 lungs contained facultative bacteria, and 30 gave no growth (Table III). Ninety-three aerobic bacterial isolates were made from the 69 lungs, but the mean number of aerobic spe-

TABLE I. Number of Anaerobic Bacteria Isolated from Different Types of Bovine Pneumonias

Total number of specimens	Type of Pneumonia	Number of specimens with anaerobes	% specimens with anaerobes
73	Acute fibrinous bronchopneumonia	24	32.8
7	Acute suppurative bronchopneumonia	2	28.5
36	Chronic suppurative bronchopneumonia	12	33.3
5	Chronic fibrinous bronchopneumonia	3	60
5	Embolic pneumonia	1	20
4	Acute interstitial pneumonia	0	0
14	Pneumonic specimens without pathological classification available	3	21.4
15	Lungs with macroscopic alterations due to various conditions (DIC, viral infection, *ductus arteriosus*)	0	0
13	Healthy lungs at slaughter	0	0

TABLE II. Anaerobic Bacteria Recovered from 45 Acute and Chronic Pneumonias of Cattle

Species	No. of isolates	Species	No. of isolates
Peptococcus indolicus	15	*Propionibacterium acnes*	2
Bacteroides asaccharolyticus	9	*Bacteroides bivius*	1
Fusobacterium necrophorum	6	*Bacteroides distasonis*	1
Bacteroides fragilis	4	*Bacteroides ruminicola* subsp. *brevis*	1
Clostridium perfringens	4	*Bacteroides ureolyticus*	1
Bacteroides intermedius	3	*Peptostreptococcus micros*	1
Bacteroides CDC group F_2	3	*Clostridium bifermentans*	1
Peptostreptococcus anaerobius	3	*Clostridium sordellii*	1
Peptococcus niger	2	*Bacteroides* spp.	6
Peptostreptococcus parvulus	2	Fusobacterium sp.	1
Peptococcus asaccharolyticus	2	*Lactobacillus* sp.	1
Clostridium butyricum	2	*Clostridium* sp.	1

TABLE III. Facultative Bacteria Recovered from Bovine Pneumonias in the Presence or Absence of Anaerobic Bacteria

Species	Number of Isolates AN[a]	AER[b]	Species	Number of Isolates AN	AER
Corynebacterium pyogenes	25[c]	15	*Proteus* species	1	1
Escherichia coli	19[d]	10	*Staphylococcus* species	1	2
Pasteurella multocida	18	23	*Moraxella* species	2	—
Pasteurella haemolytica	13	22	*Klebsiella pneumoniae*	—	1
Pseudomonas aeruginosa	6	5	*Enterobacter agglomerans*	—	1
Streptococcus species	6	6	*Mima polymorpha*	1	—
Haemophilus somnus	2	4	*Salmonella* species	1	—
Nocardia species	—	2	*Bacillus* species	—	1

[a]AN, anaerobic bacteria isolated, 45 cases
[b]AER, no anaerobic bacteria isolated, 99 cases
[c]($p<0.001$)
[d]($p<0.01$)

cies recovered did not differ significantly between lungs with and without anaerobes.

ANTIMICROBIAL SUSCEPTIBILITY OF ANAEROBIC ISOLATES

Only 37 of the anaerobic bacteria isolated grew in the commercial antimicrobial test panels used. These isolates were *P. indolicus* (15 isolates), *C. perfringens* (four), *B. fragilis* (four), *F. necrophorum* (three), *C. butyricum* (two), *P. acnes* (two), *C. sordellii* (one), *C. bifermentans* (one), *P. asaccharolyticus* (one), *P. niger* (one), *B. asaccharolyticus* (one) and two unidentified *Bacteroides* species. The cumulative susceptibility of these isolates to increasing concentrations of antimicrobial drugs is shown in Table IV.

In contrast to the commercial system, 70 of 73 isolates grew in the brain heart infustion broth used in the disc-broth method of determining antimicrobial susceptibility. The results from this semiquantitative test are shown in Table V. Penicillin G and tetracycline had the lowest activity *in vitro*; ampicillin and erythromycin showed moderate activity. Over 90% of isolates were susceptible to clindamycin, cephalothin, chloramphenicol, carbenicillin and metronidazole. Table VI shows the resistance patterns of the most frequently isolated anaerobic species, showing multiple antibiotic resistance by *C. perfringens* and *B. fragilis* isolates, and tetracycline resistance by one-third of *P. indolicus* isolates.

DISCUSSION

In this study pathogenic anaerobic bacteria were recovered in large numbers from 45 of 144 pneumonic cattle lungs, with only a slightly higher prevalence in lungs with chronic rather than active infections. The species most commonly isolated (*P. indolicus, B. asaccharolyticus, F. necrophorum*) are common as opportunist anaerobic pathogens of cattle (5, 6, 13-15). There was a significant association between the presence of *C. pyogenes* and of anaerobic bacteria, a synergistic relationship well known for *F. necrophorum* (13) and for *P. indolicus* (14). Most of the other anaerobic species recovered are causes of opportunist anaerobic infections in man (7, 8).

It is possible that some of the anaerobes isolated may have represented postmortem "overgrowth" since the cattle sampled, which were from routine submissions to a diagnostic pathology centre, included animals dead for up to 48 hours. Reason for believing that most were not the result of such "overgrowth", other than the identification of the isolates as common anaerobic pathogens of cattle and other species and the correlation with the presence of *C. pyogenes*, include the single isolation of *Proteus* species and the recovery of only nine isolates of Clostridium, both genera traditionally regarded as postmortem contaminants. Fifteen lungs with pathological changes not of bacterial origin were obtained and cultured under similar conditions to those of the bacterially infected lungs; none yielded anaerobic bacteria. Thirteen fresh lungs from healthy cattle at slaughter failed to yield bacteria.

TABLE IV. Cumulative Susceptibility of 37 Isolates of Anaerobic Bacteria to Selected Antimicrobial Drugs

Antimicrobial drug	Cumulative % Susceptible MIC (μg/mL)							
Carbenicillin	≤8[a]	16	32	64	128	256	512	>512
	91.8[b]	94.5			100			
Cefoxitin	≤1	2	4	8	16	32	64	>64
	72.9	78.3	86.4	94.5				100
Chloramphenicol	≤0.5	1	2	4	8	16	32	>32
	32.4	48.6		83.7	89.1		97.2	100
Penicillin G	≤0.06	0.12	0.25	0.5	1	2	4	> 4
	78.3		81				91.8	100
Clindamycin	≤0.25	0.5	1	2	4	8	16	>16
	86.4	91.8	97.2					100
Tetracycline	43.2	45.9	48.6	54.0	62.1	75.6	89.1	100
Metronidazole	94.5	97.2						100

[a]Top line, drug concentration, μg/mL
[b]Second line, cumulative susceptibility of isolates

No attempt was made to recover nonbacterial pathogens (viruses, mycoplasma) in this study. About half the lungs were from animals over six months of age and would thus have included cases of "shipping fever pneumonia" and half were from younger animals and would have included animals suffering from "enzootic pneumonia". Antibiotic treatment of animals before death was not recorded, but may have decreased the recovery of pathogenic bacteria.

The anaerobic bacteria in these lungs were probably present as opportunistic pathogens, secondary to a variety of predisposing factors which include host and environmental factors (1) and the action of viral and other bacterial agents. One source of these anaerobic bacteria is the rumen, as a result of the constant inhalation of large numbers of anaerobic bacteria in the eructated gases (16). The almost invariable presence of aerobic and facultatively anaerobic bacteria together with the anaerobic isolates suggested that bacterial synergistic interactions were occurring; such interactions between *C. pyogenes* and *F. necrophorum* or *P. indolicus* are recorded (13,14) and were strongly implied in this study. In man synergistic interactions have been described between *E. coli* and *B. fragilis* (17). *Bacteroides asaccharolyticus* is an important anaerobic pathogen which is only found in synergistic combination with other bacteria because of its vitamin K requirement (7).

While the mechanisms by which pathogenic anaerobic bacteria cause disease have not been well characterized, certain species can inhibit phagocytosis (18, 19), produce leukotoxins (20) or provide growth factors to stimulate other, perhaps more virulent, bacteria (13).

The finding of anaerobic bacteria in nearly one-third of the lungs of cattle at postmortem adds further complexity to our understanding of the pneumonic process in this species. Experimental work is required to delineate the role of the more commonly isolated anaerobic bacteria, and their synergistic interactions with *C. pyogenes* and *E. coli*, in pneumonia in cattle. The frequent occurrence of *C. pyogenes* in the lungs was a striking feature of the aerobic cultural findings.

The identification of the anaerobic bacteria recovered was by the Minitek method supplemented by analysis of end products of fermentation; the Minitek system has been shown to give excellent correlation with more traditional identification systems (9). In some cases GLC results were essential to differentiate between species giving similar biochemical reactions. It was necessary in the identification of *P. indolicus* since this pathogen, rarely encountered in medical bacteriology, was not identified in the "profile register" of the system. It could, however, be identified by its biochemical properties and from its distinctive fermentation end products (8).

The use of the commercial Micromedia Anaerobic MIC panel to determine MICs of the isolated organism was disappointing because of the failure of many bacteria to grow in the broth; for this reason the broth-disc method was used. Where the two systems could be prepared the results for susceptibility or resistance of the isolates tested were found to be similar. The general level of antimicrobial drug resistance of the anaerobic bacteria isolated was similar to that of human (20, 21) and animal clinical isolates (22). The tetracycline resistance observed in *P. indolicus* was higher than described previously (13). The pattern of resistance observed in *C. perfringens* has been reported previously as plasmid mediated (23). The *B. fragilis* isolates also showed multiple resistance, probably of plasmid origin.

TABLE V. Percentage Susceptibility of 70 Anaerobic Bacterial Isolates to Selected Antimicrobial Agents

Antimicrobial agent	Test concentration per mL	Percent susceptibility
Penicillin G	2 units	84.2
Ampicillin	4 μg	87.1
Carbenicillin	100 μg	98.5
Cephalothin	6 μg	92.8
Tetracycline	6 μg	84.2
Clindamycin	1.6 μg	91.4
Chloramphenicol	12 μg	92.8
Erthromycin	3 μg	87.1

TABLE VI. Resistance of Anaerobes Most Frequently Isolated from Pneumonic Cattle to Clinically Useful Antimicrobial Drugs

	Drug and Concentration Tested/mL						
Species	Penicillin 2 U	Ampicillin 4 μg	Cephalothin 6 μg	Tetracycline 6 μg	Chloramphenicol 12 μg	Clindamycin 1.6 μg	Erythromycin 3 μg
Bacteroides fragilis	4/4[a]	4/4	3/4	2/4	0/4	0/4	0/4
Peptococcus indolicus	0/15	0/15	0/15	5/15	0/15	1/15	1/15
Bacteroides intermedius	1/3	1/3	1/3	0/3	0/3	0/3	0/3
Clostridium perfringens	0/4	0/4	0/4	4/4	4/4	4/4	4/4
Fusobacterium necrophorum	0/6	0/6	0/6	0/6	0/6	0/6	3/6
Bacteroides asaccharolyticus	0/9	0/9	0/9	0/9	0/9	0/9	0/9
All other anaerobes tested	7/29	4/29	1/29	0/29	1/29	1/29	1/29

[a]Number resistance/number tested

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